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Regulation and function of V-ATPases in physiology and disease

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Abstract

The vacuolar H⁺-ATPases (V-ATPases) are essential, ATP-dependent proton pumps present in a variety of eukaryotic cellular membranes. Intracellularly, V-ATPase-dependent acidification functions in such processes as membrane traffic, protein degradation, autophagy and the coupled transport of small molecules. V-ATPases at the plasma membrane of certain specialized cells function in such processes as bone resorption, sperm maturation and urinary acidification. V-ATPases also function in disease processes such as pathogen entry and cancer cell invasiveness, while defects in V-ATPase genes are associated with disorders such as osteopetrosis, renal tubular acidosis and neurodegenerative diseases. This review highlights recent advances in our understanding of V-ATPase structure, mechanism, function and regulation, with an emphasis on the signaling pathways controlling V-ATPase assembly in mammalian cells. The role of V-ATPases in cancer and other human pathologies, and the prospects for therapeutic intervention, are also discussed.

Keywords

vacuolar ATPase; acidification; proton transport; cancer metastasis; regulated assembly; nutrient sensing

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1. V-ATPase structure and mechanism

The V-ATPase is a multi-subunit complex composed of two large domains: the soluble V_1 domain, which hydrolyzes ATP, and the membrane-embedded V_0 domain, which transports protons [1–10] (Fig. 1). V_1 is composed of subunits A through H in a stoichiometry of $A_3B_3CDE_3FG_3H$, while V_0 is composed of subunits a, c, c'', d, e, in a stoichiometry of $ac_9c''de$ (yeast also have a subunit c' in place of one of the c subunits) [2,8–10]. Eukaryotic V-ATPases are structurally and mechanistically related to the F_1F_0 ATP synthases (F-ATPases), but always perform primary active proton transport rather than using the proton motive force to drive ATP synthesis [1,2,11]. V-ATPases bear even greater similarity to the archaeal-type ATPases (A-ATPases), including the H^+ -ATPase from *T. thermophilus*, which functions in ATP synthesis, and the Na^+ -ATPase from *E. hirae*, which functions to transport sodium ions [2,12,13]. As with the F-ATPase and A-ATPase, the V-ATPase is a rotary machine, and therefore contains both stationary (stator) and rotational (rotor) subcomplexes [14–16]. The stator is made up of an A_3B_3 hexamer containing three catalytic ATP binding pockets, three peripheral EG stalks, and subunits C, H and a. The rotor is made up of a central stalk composed of subunits D and F connected to subunit d, and a proteolipid ring of subunits c, (c') and c''. While the function of subunit e is unknown, it is essential for V-ATPase activity, and its positioning suggests it is a stator subunit [17–20].

ATP hydrolysis causes conformational changes in the catalytic hexamer which drive rotation of the central stalk and the proteolipid ring [16,21–23]. Rotation of the proteolipid ring relative to subunit a is essential for proton transport [24]. This is because subunit a contains two aqueous hemichannels: a cytosol-facing hemichannel enables proton entry, while a lumen-facing hemichannel enables proton exit. As protons enter from the cytosol, they protonate a buried glutamate residue contained in each proteolipid subunit [25–28]. Rotation of the proteolipid ring causes the protonated glutamates to sequentially encounter a critical buried arginine residue in subunit a, enabling proton release and exit through the lumen-facing hemichannel [29–31].

The peripheral stator subunits, consisting of three EG stalks (designated EG1, EG2 and EG3) [32] and subunits C, H and a, stabilize the entire complex and prevent rotation of the stator during ATP hydrolysis. The EG C-termini bind the N-termini of the B subunits, while the EG N-termini bind to subunits C, H and the N-terminus of subunit a (a_{NT}) [33–36]. Subunits C and H are each composed of two globular domains (C_{head}/C_{foot} and H_{NT}/H_{CT}) connected by a flexible linker [37,38]. C_{head} interacts strongly with EG3 in a binary interface [39], while C_{foot} interacts relatively weakly with EG2 and a_{NT} in a ternary interface [40]. Another relatively weak ternary interaction is formed by subunit H, EG1, and a_{NT} [41]. Upon disassembly (discussed below), subunit H undergoes a substantial conformational shift, bringing H_{CT} in contact with subunit F of the central stalk, possibly to prevent rotation [42–44]. Moreover, a crystal structure of the yeast V_1 has revealed that H_{CT} may inhibit ATP hydrolysis by stabilizing one of the catalytic sites in the open state [45].

Additional putative V-ATPase subunits have recently been identified. A 3.9-Å cryo-EM structure of yeast V_0 revealed the existence of an integral protein associated with subunit a and adjacent to subunit e, which has been designated subunit f [27]. Furthermore, a recently

resolved 3.5-Å cryo-EM structure of yeast V_0 revealed the presence of V_0 assembly protein 1 (Voa1) within the center the proteolipid ring [28]. Voa1p functions in biosynthetic assembly of the V-ATPase in the endoplasmic reticulum, and was recently proposed to be the yeast homolog of Ac45, a protein which also functions in V-ATPase biosynthesis in higher eukaryotes [47–49]. Ac45 also associates with certain assembled V-ATPase complexes, and appears to be important for acidification of secretory vesicles in neuroendocrine cells, and for osteoclast function [50,51]. Proteins such as Ac45, which are not exclusively found within V-ATPase complexes, are classified as V-ATPase accessory subunits. Another accessory subunit present in higher eukaryotes (but not yeast) is the prorenin receptor (PRR), which associates with V_0 , and this interaction appears to be important for Wnt signaling [52].

2. V-ATPase function

2.1. Function of intracellular V-ATPases

Luminal pH, which is chiefly controlled by V-ATPase-dependent proton transport, is a critical parameter for the function of various organelles. Thus, the acidic pH within sorting endosomes triggers the release of ligands internalized via receptor-mediated endocytosis, enabling the return of unoccupied receptors to the cell surface. Receptor recycling is important for controlling the rate of uptake of ligands such as low-density lipoprotein and transferrin [53]. Rates of recycling can also control the cell surface density of particular receptors, thus influencing the sensitivity of responses to hormones and growth factors such as insulin and epidermal growth factor (EGF). Dissociation from receptors causes signal termination and enables internalized ligands to reach their final cellular destinations, usually culminating in lysosomal degradation. Another type of receptor recycling occurs in late endosomes, where acidic pH triggers the release of newly synthesized proteases from the mannose 6-phosphate receptor (MPR). Ligand-receptor dissociation in this case enables both protease delivery to lysosomes and the return of MPRs to the *trans*-Golgi network [54]. Endosomal acidification also plays a role in the formation of certain carrier vesicles, which transport cargo within the endocytic and secretory pathways [55].

Several pathogens take advantage of the low pH within endosomes to gain entry into the cytoplasm. Exposure to acidic pH facilitates fusion of enveloped viruses such as Influenza and Ebola with the endosomal membrane, which is required for the insertion of viral genomes into the cytosol [56]. Endosomal pH also promotes the entry of pathogenic agents such as diphtheria toxin, which enters from sorting endosomes, and anthrax toxin, which first enters multivesicular bodies and then is released from late endosomes [57]. Certain secretory vesicles are acidified by the V-ATPase to facilitate the proteolytic processing of prohormones such as proinsulin [58]. Proteolytic processing also takes place in dendritic cell lysosomes, where internalized antigens are packaged for presentation on major histocompatibility complex class II molecules [59]. The proton gradient or membrane potential imposed by the V-ATPase is also coupled to small molecule transport. Thus, the positive interior membrane potential within synaptic vesicles drives glutamate uptake, while noradrenaline and GABA are loaded into synaptic vesicles by proton/neurotransmitter

antiporters [2,60]. In lysosomes, several types of proton/amino acid symporters use the proton gradient to drive amino acid efflux [61].

Because most lysosomal hydrolases require acidic pH for activity, the V-ATPase is essential for proper degradation of macromolecules [62]. Macromolecules are delivered to lysosomes either endocytically, via chaperone-mediated autophagy, or via macroautophagy (hereafter autophagy), a catabolic program for recycling cellular components [63,64]. During autophagy, organelles and other macromolecules are engulfed within double-membraned vesicles called autophagosomes. Autophagosomes eventually fuse with lysosomes to form autolysosomes, in which the luminal contents are degraded, and the resulting catabolites returned to the cytosol to be reused [65]. During this process, acidification is essential for both autophagosome/lysosome fusion and for subsequent breakdown of luminal contents [66–68]. In fact, V-ATPase inhibitors such as bafilomycin A1 are such potent blockers of autophagic flux that they are often used as autophagy inhibitors [69]. Autophagy normally operates at low basal levels but can be substantially upregulated during times of energy stress or nutrient deprivation [70]. In addition, autophagy is an important survival mechanism employed by cancer cells (discussed below).

2.2. Function of plasma membrane V-ATPases

V-ATPases are localized to the plasma membrane of certain polarized animal cells where they function to transport protons to the extracellular space. These include osteoclasts, α -intercalated cells of the kidney collecting duct, clear cells of the epididymis, interdental cells of the inner ear and sustentacular cells of the olfactory epithelium [2,5]. In osteoclasts, plasma membrane V-ATPases function in bone resorption, which depends on acidification of the osteoclast/bone interface. Mutations in subunit a3, which targets the V-ATPase to the plasma membrane of osteoclasts, lead to a severe congenital form of osteopetrosis in humans [71,72]. Renal α -intercalated cells possess pools of V-ATPase-rich vesicles poised for fusion with the apical membrane [2,73]. These cells respond to alterations in plasma pH by rapidly adjusting the density of apical V-ATPases, which function to acidify urine, thereby removing excess acid from the blood. Mutations in subunit a4, which targets the V-ATPase to the plasma membrane of intercalated cells, cause recessive distal renal tubular acidosis in humans [74,75]. A similar form of endocytosis/exocytosis of V-ATPase-rich vesicles occurs in the clear cells of the epididymal epithelium. Acidification of the epididymal fluid maintains spermatozoa in a quiescent state, which is necessary for proper maturation and storage [2,76]. While V-ATPase mutations have not been directly linked decreased fertility in humans, male mice engineered to lack plasma membrane V-ATPase expression in the epididymis have elevated epididymal fluid pH, defective sperm, and are infertile [77]. Finally, V-ATPases have been detected at the plasma membrane in numerous types of human cancers, where they appear to aid in tumor cell invasiveness [78]. A detailed discussion of V-ATPases in cancer is presented in the section on V-ATPases in disease.

3. V-ATPase regulation

3.1. Regulated assembly

Because so many essential processes occur within discrete pH ranges, V-ATPase activity is highly regulated. Among the most important forms of V-ATPase regulation is the reversible dissociation of the V-ATPase complex into its component V_1 and V_0 domains, a process termed regulated assembly (Fig. 2). In the dissociated state, both the ATP-hydrolytic function of V_1 and the proton transport function of V_0 are inhibited (as discussed in the section on V-ATPase structure and mechanism) [2,79–82]. Since ATP consumption is prevented in the dissociated state, disassembly has been proposed to be a mechanism for conserving energy during times of nutrient limitation. Thus, the first demonstrations of regulated assembly were in insect cells during molting and yeast cells subjected to glucose starvation [83,84].

Regulated assembly enables rapid and local adjustment of V-ATPase activity. In yeast, for example, glucose starvation causes disassembly of V-ATPases localized to the vacuole [83,85], while Golgi-localized V-ATPases remain assembled [86]. When adequate glucose is present, yeast cells maintain V-ATPase assembly through activation of the Ras/adenylate cyclase/cAMP-dependent protein kinase (PKA) pathway [87]. Interestingly, elevated glucose was found to increase cytosolic pH in yeast, and to promote PKA activity in a V-ATPase-dependent manner, suggesting the existence of a positive feedback loop between PKA and the V-ATPase in yeast [88]. Assembly also responds to alterations in extracellular and vacuolar pH. In yeast grown at elevated pH or treated with chloroquine to neutralize the vacuole, V-ATPase disassembly upon glucose starvation is reduced [89,90].

While PKA controls V-ATPase assembly in response to glucose availability in yeast, it is also involved in controlling assembly in insect cells. In blowfly salivary glands, pharmacological elevation of intracellular cAMP leads to PKA-dependent phosphorylation of subunit C and increased V-ATPase assembly [91–93]. These results suggest that direct phosphorylation of V-ATPase subunits may be involved in regulating assembly, although this has not yet been directly demonstrated. Interestingly, recent FRET experiments in yeast found that glucose starvation leads to dissociation of subunit C from the entire V-ATPase complex, while the rest of V_1 remains in close proximity with the membrane [94]. This finding suggests that association of subunit C may be a key determining factor in the stability and activity of V-ATPases *in vivo*. Another region that appears to be important for controlling assembly is the non-homologous region (NHR) of subunit A, so-named because it is a 100 amino acid insert not present in the otherwise homologous F-ATPase β subunit [1,2,95]. Introduction of specific mutations in this region was found to block disassembly upon glucose starvation without altering catalytic activity [96]. Remarkably, in cells expressing only a NHR-hemagglutinin fusion protein in place of the endogenous A subunit, the NHR was found to associate with the V_0 domain in a glucose-dependent manner [90].

Disassembly and reassembly appear to be controlled by different mechanisms. In yeast subjected to glucose starvation, disassembly – but not reassembly – requires an intact microtubule network [97]. Disassembly also requires the V-ATPase to be catalytically active, suggesting the enzyme must be able to enter a particular conformation to undergo

dissociation [85,98,99]. Reassembly – but not disassembly – is mediated by a number of proteins referred to as assembly factors. These include the glycolytic enzymes aldolase and phosphofructokinase, which physically interact with the V-ATPase in a glucose-dependent manner and function to promote assembly [100,101]. Another important assembly factor is Regulator of H⁺-ATPase of vacuoles and endosomes (RAVE), which is required for proper V-ATPase reassembly when cells are returned to normal glucose levels [102,103]. RAVE is believed to act as a chaperone to facilitate reintegration of free C subunits within the V₁ subcomplex [104]. Furthermore, RAVE is recruited to vacuolar membranes by Vph1p during glucose sufficiency and is released to the cytosol during starvation, highlighting the dynamic nature of the RAVE/V-ATPase interaction [105]. Interestingly, RAVE displays specificity with respect to V-ATPase isoforms, by promoting assembly of Vph1p-containing, but not Stv1p-containing complexes [106]. In *Drosophila* and mammals, Rabconnectin-3 α (Rbcn-3A) is homologous to Rav1p, and is important for endosomal acidification and Notch signaling [107,108].

Yeast also increase V-ATPase assembly in response to salt stress and this change is mediated by increased levels of PI(3,5)P₂, a predominantly vacuolar inositol lipid produced by the phosphatidylinositol 3-phosphate 5-kinase Fab1p (homologous to mammalian PIKfyve) [109,110]. Interestingly, glucose-mediated assembly changes are independent of PI(3,5)P₂ levels [109]. PI(3,5)P₂ was found to preferentially bind to the N-terminal domain of Vph1p and increase V-ATPase catalytic activity and proton pumping [109,111]. Alternatively, PI(4)P binds to the N-terminal domain of Stv1p, and this interaction appears to be important for proper targeting of Stv1p-containing V-ATPases to the Golgi [112]. Intriguingly, PI(4)P interacts with the N-terminal domain of human $\alpha 2$, suggesting lipid interactions may represent a conserved mechanism for controlling V-ATPase trafficking and possibly activity [112].

Regulated assembly occurs in mammalian cells in response to diverse stimuli. Assembly increases in cells exposed to elevated glucose concentrations in a phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)-dependent manner, perhaps as a way to contend with excess glycolytic acid generation [113,114]. Assembly increases in cells infected with Influenza virus in both a PI3K and mitogen-activated protein kinase (MAPK)-dependent pathway in order to acidify endosomes and promote viral fusion [115]. Recently, PI3K, Akt and MAPK1/2 were found to physically associate with subunit E to promote assembly and endosomal acidification in cells infected with rotavirus [116]. Further investigation will be needed to determine whether direct association of these important kinases represents a common mechanism by which assembly is controlled in mammalian cells. PI3K and mTOR complex I (mTORC1) are also important for increased lysosomal assembly in dendritic cells exposed to a maturation stimulus, where enhanced lysosomal acidification promotes antigen processing [117,118]. Assembly also increases in cells exposed to EGF, and V-ATPase inhibition was shown to prevent EGF-stimulated mTORC1 activation [119]. Finally, increasing evidence points to lysosomal V-ATPase assembly functioning in cellular nutrient homeostasis. Glucose starvation rapidly increases V-ATPase assembly and lysosomal acidification in mammalian cells in a PI3K and 5'-AMP-activated protein kinase (AMPK)-dependent manner [120]. Amino acid starvation also increases assembly and lysosomal acidification, and this effect was found to be PI3K-independent [121]. A more detailed

discussion of the V-ATPase in nutrient sensing can be found in the section on emerging functions of the V-ATPase.

3.2. Regulated trafficking

Another important form of V-ATPase regulation is the targeting of V-ATPases to different cellular membranes, which is controlled by isoforms of subunit a (also discussed in V-ATPase structure and mechanism). Subunit a is the only V-ATPase subunit with multiple isoforms in yeast, where it is encoded by the genes *VPH1* and *STV1* [125,126]. Both Vph1p and Stv1p contain targeting information within their N-termini, with Vph1p-containing V-ATPases being targeted to the vacuole, while Stv1p-containing V-ATPases are targeted to the Golgi [127]. In mammals, subunit a exists in four isoforms encoded by the genes *ATP6V0A1*, *ATP6V0A2*, *TCIRG1* and *ATP6V0A4* [128]. a1 and a2 are primarily found within intracellular membranes, although a1 is also targeted to presynaptic plasma membranes of nerve terminals [124,129,130]. a3 is found intracellularly in compartments such as lysosomes, phagosomes and secretory granules, and is also targeted to the plasma membrane of osteoclasts [131–134]. While undifferentiated osteoclast progenitor cells localize a3 to late endosomes and lysosomes, these a3 containing-V-ATPases are trafficked to the plasma membrane upon differentiation into mature osteoclasts [131]. a1-a3 are expressed in a broad range of tissues, whereas a4 appears to be restricted to the kidney, epididymis and inner ear [71,75,135,136]. a4-containing V-ATPases are localized to the apical plasma membrane of renal intercalated cells and epididymal clear cells where they function in urinary acidification and sperm maturation, respectively [75,136].

In addition to isoform composition, which targets V-ATPases to particular membranes, certain polarized cells are able to rapidly alter the density of V-ATPases at the plasma membrane. In both renal intercalated cells and epididymal clear cells, exposure to alkaline pH leads to increased bicarbonate uptake and activation of PKA via a bicarbonate-sensitive adenylate cyclase [5]. cAMP stimulates insertion of V-ATPases into the apical membrane of these cells, and PKA-dependent phosphorylation of subunit A appears to be important for this process [137–139]. Conversely, AMPK was found to block PKA-induced apical accumulation of the V-ATPase in renal and epididymal cells [140]. In kidney cells, this was found to depend on direct phosphorylation of subunit A by AMPK [141]. It should be noted that these studies used localization of V₁ subunits as a surrogate for trafficking of the intact enzyme, so it is possible that AMPK and PKA also influence V-ATPase assembly in these cell types.

3.3. Other forms of regulation

Several other forms of V-ATPase regulation have been observed, including disulfide bond formation, changes to coupling efficiency, changes in counterion conductance and regulated expression of V-ATPase subunits. In bovine brain clathrin-coated vesicles, approximately 50% of V-ATPases were found to be inactive, due to disulfide bonding between C254 and C532 in the catalytic A subunit [142,143]. This inhibition can be relieved by disulfide interchange with a third conserved cysteine residue [144].

Another possible mechanism for regulating V-ATPase activity *in vivo* is by changing the ratio of proton transport to ATP hydrolysis, i.e., the coupling efficiency. Certain point mutations in the non-homologous region of subunit A were shown to increase the coupling efficiency, suggesting that wild-type yeast V-ATPases may not be optimally coupled [96]. Furthermore, coupling is influenced by isoforms of subunit a, with Vph1p-containing V-ATPases being 4–5 times more efficiently coupled than Stv1p-containing V-ATPases [86]. A recent report found that the entrance to the cytosolic hemichannel in subunit a has a negative surface charge in Vph1p, and a positive surface charge in Stv1p [46]. Future studies will be needed to determine whether this property contributes to the observed differences in coupling efficiency between Vph1p- and Stv1p-containing V-ATPases.

Another way that cells may alter V-ATPase activity in particular compartments is through counterion conductance [145]. Chloride channels help to dissipate the interior positive membrane potential generated by the V-ATPase. The H⁺/Cl⁻ antiporter ClC-5 is present in V-ATPase-rich apical vesicles in renal intercalated cells, and *CLC5* mutations cause Dent's disease, which is characterized by defective endosomal acidification and kidney failure [146]. The major lysosomal H⁺/Cl⁻ antiporter ClC-7 was found to be important for the ability of primary mouse microglia to degrade amyloid- β (A β) – a causative agent of Alzheimer's disease (discussed below) [147]. Interestingly, plasma membrane ClC7 is also important for osteoclast function, and *CLC7* mutations result in osteopetrosis [148].

4. V-ATPases in disease

4.1. Cancer

There is considerable evidence that V-ATPases contribute to the survival and spread of cancer cells through several mechanisms (Fig. 3). One of the ways that V-ATPases have been proposed to promote tumor cell survival is by maintaining a neutral cytosolic pH. Owing to a combination of hypoxia and increased glycolytic metabolism, tumor cells are forced to contend with elevated levels of cytosolic acid [149]. It is believed that cancer cells increase V-ATPase biosynthesis and targeting of V-ATPases to the plasma membrane in order to secrete this acid extracellularly [150]. Plasma membrane V-ATPase localization has been detected in human samples of breast and lung tumors, and numerous cancer cell lines, including Ewing sarcoma, melanoma, breast, liver, pancreatic, prostate and ovarian cancer [151–159]. Indeed, tumors often exhibit a reversed pH gradient, with extracellular pH within the tumor microenvironment being lower than the intracellular pH of the tumor cells [160]. V-ATPase inhibitors have been shown to induce apoptosis in several cancer cell lines, possibly by blocking acid extrusion [161–164].

V-ATPases also promote cancer cell survival by preventing cancer drugs from reaching their intended targets. Many chemotherapeutic agents are weak bases that can become protonated in the weakly acidic tumor microenvironment [165]. Protonation in the extracellular environment may prevent drugs from entering cancer cells, and numerous studies have found that raising extracellular pH increases drug uptake, cytosolic retention and tumor cell killing [166–168]. This problem is also enhanced by the fact that drugs in the more alkaline cancer cell cytoplasm are deprotonated and can diffuse out of the cell or partition into acidic compartments [169,170]. These resistance mechanisms may partially explain the enhanced

efficacy observed by combining chemotherapeutics with V-ATPase inhibitors [171–174]. Other novel mechanisms promoting cancer cell growth and survival may be tumor type-dependent. A recent study found V-ATPase inhibition stabilized Hypoxia-inducible factor 1-alpha (HIF1 α) by preventing its iron-dependent hydroxylation and degradation [175]. Stabilized HIF1 α translocates to the nucleus where it represses expression of androgen receptor, which is drives the growth and survival of prostate tumors [175].

In addition to cancer cell survival, a number of studies have suggested that V-ATPases function to promote invasiveness, which is a fundamental property of metastatic cells. While metastasis is the leading cause of death from cancer, current cancer therapies are not specifically designed to prevent metastasis [176]. In order to metastasize, cells must detach from the primary tumor, penetrate basement membrane to enter the vascular or lymphatic systems, and then extravasate to colonize distant sites [177]. Several of these steps require cleavage of extracellular matrix components by secreted proteases such as cathepsins and matrix metalloproteases. Cathepsins have been found to be overexpressed in many human cancers and are correlated with worse prognosis [178]. Encouragingly, pharmacological and genetic inhibition of cathepsins reduced the spread of metastatic breast cancer models in mice [179,180]. Secreted cathepsins are thought to be activated within partially sealed extracellular spaces that are acidified by plasma membrane V-ATPases. Pan-V-ATPase inhibition with membrane-permeant inhibitors is highly effective at reducing *in vitro* cancer cell migration and/or invasion [151,155,181–187]. While these studies suggest V-ATPases are important for tumor cell invasiveness, they do not differentiate between intracellular and plasma membrane V-ATPases. Moreover, pan-V-ATPase inhibition would likely be too toxic in humans to be therapeutically viable, unless the drug could be selectively delivered to tumor cells.

As described above, V-ATPases have been detected at the plasma membrane of numerous invasive cancer cell lines. Furthermore, plasma membrane V-ATPase localization is significantly higher in invasive MCF10CA1a cells compared to noninvasive parental MCF10a cells [183]. Since plasma membrane targeting is controlled by isoforms of subunit a, it is likely that cancer cells will upregulate particular isoforms in order to increase localization of V-ATPases to the plasma membrane. Invasive MDA-MB-231 and MCF10CA1a cells markedly upregulate a3 relative to non-invasive MCF7 and MCF10a cells, respectively [182,183]. Importantly, RNAi-mediated knockdown of a3 significantly reduces invasion of MDA-MB-231 and MCF10CA1a cells, and overexpression of a3 in MCF10a cells significantly increases their invasion and plasma membrane V-ATPase localization [182,183]. To test whether specific inhibition of plasma membrane V-ATPases is sufficient to reduce invasion, MDA-MB-231 cells were engineered to express the V5 epitope fused to subunit c, such that V5 is oriented extracellularly in plasma membrane V-ATPases. Treatment of c-V5-expressing cells with an anti-V5 antibody inhibited plasma membrane V-ATPase activity and significantly reduced invasion [184]. Similarly, selective inhibition of cell surface V-ATPases using the membrane impermeant reagent biotin-bafilomycin bound to streptavidin inhibited *in vitro* invasion to the same extent as concanamycin, a membrane permeant inhibitor, arguing that inhibition of plasma membrane V-ATPases is the critical target in reducing tumor cell invasion (157). Furthermore, using isoform-specific antibodies, the invasive lines MDA-MB-231, SUM149 and MCF10CA1a were found to localize more

α 3-containing V-ATPases to the plasma membrane than non-invasive MCF10a cells [151]. Moreover, examining 42 human breast cancer cDNA samples revealed α 3 to be upregulated in all samples, from 2.5 to nearly 50-fold [151]. Finally, immunofluorescence of human tumor samples showed α 3 to be upregulated in regions of invasive breast carcinoma relative to adjacent non-invasive tumors and normal tissue [151]. Encouragingly, specific inhibition of α 3-containing V-ATPases reduces metastases in the B16 murine melanoma model [154].

In addition to α 3 other α isoforms have been observed at the plasma membrane of certain cancer cells. α 1 and α 3, but not α 4 were detected at the plasma membrane of PC-3 prostate cancer cells and siRNA-mediated knockdown of either isoform reduced *in vitro* invasion [158]. α 2 has been detected at the plasma membrane of several different ovarian cancer cell lines [159]. Finally, a recent study using CRISPR/Cas9 to target α -isoform genes found α 4 to be critical for *in vitro* invasion of metastatic murine 4T1 breast cancer cells [185]. In this cell line, only knockout of α 4 reduced plasma membrane V-ATPase localization and *in vitro* invasion [185]. These studies suggest that plasma membrane V-ATPases represent an important and novel target in the development of new therapeutics to reduce cancer metastasis. Orthotopic implantation of tumor cells engineered to reduce plasma membrane V-ATPase activity in mice and the development of inhibitory antibodies directed against extracellular epitopes on the V-ATPase will test the hypothesis that plasma membrane V-ATPases promote breast cancer metastasis *in vivo*.

It should be noted that many of the studies showing a role for the V-ATPase in promoting cancer cell invasion have also found it to be important for cell motility. While it is still unclear how V-ATPases function in cell migration, one possible mechanism is through actin rearrangement. In normal cells, subunits B and C directly interact with actin microfilaments [188,189]. Moreover, V-ATPases colocalize with F-actin at the leading edge of microvascular endothelial cells and bafilomycin treatment reduced their *in vitro* migration [190]. A similar pattern of colocalization between V-ATPases and F-actin has been observed at the leading edge of migrating cancer cells [151,182,185,187]. Treatment of HeLa cells with the novel V-ATPase inhibitor iejimalide C, or prostate cancer cells with bafilomycin or concanamycin disrupted actin filament organization, while knockdown of subunit C had a similar effect in breast cancer cells [187,191,192]. It is thought that cell surface V-ATPases contribute to invadopodia formation, and thus cell migration, by creating a pH and/or electrochemical gradient favorable to actin polymerization and branching. Alkaline pH activates the actin binding protein cofilin, which severs actin filaments to create free barbed ends for polymerization and membrane protrusion [149]. Alternatively, certain plasma membrane calcium channels are activated at low extracellular pH, with the resulting calcium influx activating myosin light chain kinase, leading to force-generation at the leading edge [193].

4.2. Neurodegenerative diseases

A pathological hallmark of many neurodegenerative disorders is the toxic buildup of undegraded protein aggregates in the brain. Alzheimer's disease, for example, is associated with the deposition of extracellular plaques made of insoluble A β [194]. Mutations in Presenilin-1 (PS1), a component of the γ -secretase complex responsible for amyloid

processing, cause familial Alzheimer's disease [195]. Defects in PS1 prevent proper a1 trafficking and cause impaired lysosomal acidification, while rescue of lysosomal acidification in PS1 knockout cells reduced A β burden [196]. Another major neurodegenerative disorder linked to toxic protein aggregation is Parkinson's disease, in which α -synuclein aggregates are deposited in neuronal inclusions called Lewy bodies [197]. Mutations in *ATP6AP2*, which encodes the V-ATPase accessory subunit Ac45, cause X-linked Parkinsonism with spasticity, an early-onset form of Parkinsonism with defective lysosomal acidification [198]. Many other neurodegenerative disorders which are not directly caused by V-ATPase defects nevertheless exhibit lysosomal impairment [199]. Restoration of lysosomal function therefore represents an attractive therapeutic concept that should be investigated further.

4.3. Other genetic disorders

As discussed above, mutations in V-ATPase subunit genes can result in dysfunction of particular cell types. Mutations in a3, which targets the V-ATPase to the plasma membrane of osteoclasts, cause osteopetrosis, which is characterized by dense and brittle bones [72]. In cells expressing a3^{R444L}, which causes a severe form of infantile osteopetrosis, a3 is unglycosylated, retained in the ER and degraded [200]. Mutations in a4 and B1, which are present in plasma membrane V-ATPases in the kidney and cochlea, lead to recessive distal renal tubular acidosis and sensorineural deafness [74,201,202]. In cells expressing the a4^{R449H} mutation, a4 is retained in the ER and degraded [203]. a4 also targets the V-ATPase to the apical membrane of epididymal clear cells, but fertility information is not yet available for patients with a4 mutations [5]. Mutations in a2, which targets the V-ATPase to Golgi and endosomes, cause autosomal recessive cutis laxa type II, a rare connective tissue disorder associated with widespread glycosylation defects [204]. In cells expressing the a2^{P405L} mutation, a2 failed to traffic to the Golgi and was rapidly degraded [203].

5. Emerging functions of the V-ATPase

5.1. Amino acid sensing

Lysosomal membranes contain a nutrient-sensing supercomplex consisting of the V-ATPase, the Ragulator complex and the Rag GTPases (Fig. 4A). The Rag GTPases respond to amino acid levels by adopting a conformation which promotes binding of mTORC1 (via its Raptor subunit) when amino acids are abundant [205–207]. Upon recruitment to the lysosome, mTORC1 contacts the GTPase Rheb, which in turn is regulated by growth factor signaling [208]. Growth factors activate the PI3K pathway, leading to activation of Akt, which phosphorylates and inhibits Tuberous sclerosis complex 2 (TSC2) [209]. TSC2 is a Rheb GAP, so inhibition of TSC2 enables Rheb to activate mTORC1 when it is present at the lysosome [210]. mTORC1 activation promotes anabolic processes such as translation and ribosome biogenesis, and inhibits catabolic processes such as autophagy [211]. By integrating both amino acid availability and growth factor signaling, mTORC1 promotes growth only when adequate nutrients are present. Interestingly, pharmacological and genetic disruption of the V-ATPase inhibits amino acid-dependent mTORC1 activation [212]. In a separate study, amino acids were found to regulate the V-ATPase, with acute amino acid withdrawal leading to increases in V-ATPase assembly and V-ATPase-dependent lysosomal

acidification [121]. While this finding may be suggestive of V-ATPase assembly playing a role in regulating mTORC1, other findings rule out that possibility. First, time-course experiments show that mTORC1 inhibition precedes V-ATPase assembly changes in response to amino acid starvation [121]. Second, removal of particular individual amino acids have differential effects on V-ATPase assembly and mTORC1 activation [121]. Third, while lysosomal neutralization with chloroquine blocks assembly changes in response to amino acid starvation and also inhibits mTORC1, it does not prevent mTORC1 activation upon amino acid readdition [121,212]. These findings suggest that V-ATPase assembly is not involved in transmitting amino acid availability to mTORC1. Instead, enhanced lysosomal acidification upon amino acid starvation represents a mechanism to rapidly increase protein turnover in times of nutrient stress. Interestingly, while other examples of regulated assembly in mammalian cells depend upon PI3K (discussed above), assembly changes in response to amino acid starvation were unaffected by PI3K inhibition [121]. Thus, regulated assembly of the V-ATPase appears to be a novel mechanism involved in amino acid homeostasis. Future studies will focus on identifying the signaling pathways controlling assembly in response to amino acid starvation and measuring the degree to which this process contributes to increased lysosomal acidification and protein degradation in living cells.

5.2. Glucose Sensing

As with mTORC1, the lysosomal membrane is a platform for AMPK activation. In the canonical model for AMPK activation, AMPK senses cellular AMP:ATP ratios, such that during energy-poor conditions it is able to be activated by liver kinase B1 (LKB1) [213]. Interestingly, another form of AMPK regulation has been discovered, in which AMPK directly responds to glucose levels. During glucose starvation, LKB1 and the scaffolding protein Axin are recruited to the lysosomal membrane, leading to AMPK activation (and mTORC1 displacement) [214] (Fig. 4B). The V-ATPase is required for this process, as RNAi-mediated knockdown of subunit c blocked glucose-dependent translocation of Axin-LKB1 to the lysosome [214]. Interestingly, aldolase appears to be important for this process, as addition of the aldolase glycolytic substrate fructose-1,6-bisphosphate induces dissociation of Axin-LKB1 from the V-ATPase [215]. It was also found that treatment of cells with the V-ATPase inhibitor concanamycin promoted LKB1 recruitment even in unstarved cells [214]. This finding suggests that the V-ATPase inhibitor concanamycin (which prevents conformational changes of the V-ATPase complex) may lock the enzyme in a state that mimics the glucose-starved conformation. While glucose starvation is known to decrease assembly in yeast, a recent study found that acute glucose starvation increases assembly and V-ATPase-dependent lysosomal acidification in mammalian cells [120]. While rapid and reversible, increased assembly upon glucose starvation is preceded by AMPK activation, indicating changes in assembly are not required for glucose-dependent AMPK activation [120]. Furthermore, pharmacological inhibition of either AMPK or PI3K blocked the glucose starvation-dependent increases in V-ATPase assembly and lysosomal acidification, suggesting that these kinases are important for this effect [120]. Future studies will focus on understanding the mechanism by which these signaling pathways modulate V-ATPase activity and testing the hypothesis that cells increase V-ATPase assembly during glucose starvation to promote the utilization of energy sources derived from autophagy.

5.3. Wnt and Notch signaling

The V-ATPase and its accessory subunit PRR appear to be required for full activation of the Wnt signaling pathway. Upon Wnt binding, Frizzled receptors and their coreceptors LDL receptor-related protein 5/6 (LRP5/6) are internalized into signaling endosomes. Here, PRR acts as an adapter between the receptor complex and the V-ATPase. V-ATPase activity appears to be important for LRP5/6 phosphorylation and therefore propagation of the signal [52]. The mechanism by which V-ATPase activity promotes LRP5/6 phosphorylation and activation is still unknown. In Notch signaling, the Notch receptor undergoes a series of cleavage events which release the Notch intracellular domain for translocation to the nucleus. V-ATPase activity appears to be important for cleavage following internalization of the Notch receptor [107]. Interestingly, in *Drosophila*, Rbcn-3A (homolog of the yeast RAVE subunit Rav1p) was also required for proper Notch trafficking and signal transduction [108].

6. Conclusions and future directions

V-ATPases are essential enzymes with diverse physiological roles, which has led to the evolution of multiple forms of regulation. These include regulated assembly of the peripheral and integral domains, regulated trafficking of assembled complexes and differential properties of complexes based on their isoform composition. A remarkable property of the V-ATPase is the rapidity with which assembly (and thus activity) can be adjusted. Biochemical and genetic studies have begun to elucidate the signaling pathways controlling assembly in response to various stimuli, but many questions still remain. For instance, while protein kinase pathways important for controlling assembly have been identified, the mechanism by which these kinases alter assembly is still unknown. Furthermore, the role of the V-ATPase in nutrient sensing is only beginning to come into focus. It is clear that the V-ATPase is an essential component of a lysosomal signaling hub that functions to balance anabolic and catabolic processes. More studies will be needed to conclusively demonstrate that regulated assembly is a homeostatic mechanism to rapidly adjust lysosomal pH, and thus macromolecule degradation, in response to nutrient availability. A greater understanding of how lysosomal pH is controlled may enable development of novel therapies for diseases characterized by the failure to clear toxic protein aggregates.

A particularly promising area of research is the role of V-ATPases in cancer. There is now considerable evidence that V-ATPases contribute to the survival and invasion of numerous types of human cancer cells. While pan-V-ATPase inhibition with membrane permeant small molecules would likely be too toxic to humans, studies suggest that invasive cell lines and at least some human tumors markedly overexpress a subunit isoforms that target the V-ATPase to the plasma membrane [151,154,182–185]. Furthermore, inhibition of only plasma membrane V-ATPases with either a monoclonal antibody or a membrane-impermeant form of bafilomycin reduced the *in vitro* invasion of highly invasive breast cancer cells to the same degree as pan-V-ATPase inhibition [184]. Since few normal cell types localize V-ATPases to the plasma membrane, this type of therapy would be inherently tumor-targeted, and likely would have minimal side effects. An added benefit of plasma membrane V-

ATPase inhibition would be that such a therapy would likely inhibit osteoclast function and thus bone metastasis. As there are currently no therapies that specifically target the metastatic process, plasma membrane V-ATPases are therefore highly novel targets with the potential to be extremely impactful in the treatment of cancer.

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Abbreviations

A-ATPase	archaeal-type ATPase
Ac45	V-type proton ATPase subunit S1
Akt	cellular homolog of v-Akt, also known as RAC and PKB
AMPK	5'-AMP-activated protein kinase
EGF	epidermal growth factor
F-ATPase	F ₁ F ₀ ATP synthase
LKB1	liver kinase B1
MAPK	mitogen-activated protein kinase, also known as ERK
mTORC1	mTOR complex I
NHR	non-homologous region
PI3K	phosphatidylinositol 4,5-bisphosphate 3-kinase
PKA	cAMP-dependent protein kinase
PRR	prorenin receptor
RAVE	regulator of H ⁺ -ATPase of vacuoles and endosomes
Stv1p	V-type proton ATPase subunit a, Golgi isoform
TSC2	tuberous sclerosis 2 protein, also known as Tuberin
V-ATPase	vacuolar H ⁺ -ATPase
Voa1	V0 assembly protein 1
Vph1p	V-type proton ATPase subunit a, vacuolar isoform

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BBAMEM-20-104 Highlights

- Regulated assembly is a major mechanism for controlling V-ATPase activity *in vivo*
- The V-ATPase is part of a nutrient sensing supercomplex on the lysosomal surface
- V-ATPases are upregulated in cancer and promote tumor cell survival and metastasis
- Plasma membrane V-ATPases represent promising and novel cancer therapeutic targets
- Understanding lysosomal pH control may lead to new treatments for neurodegeneration

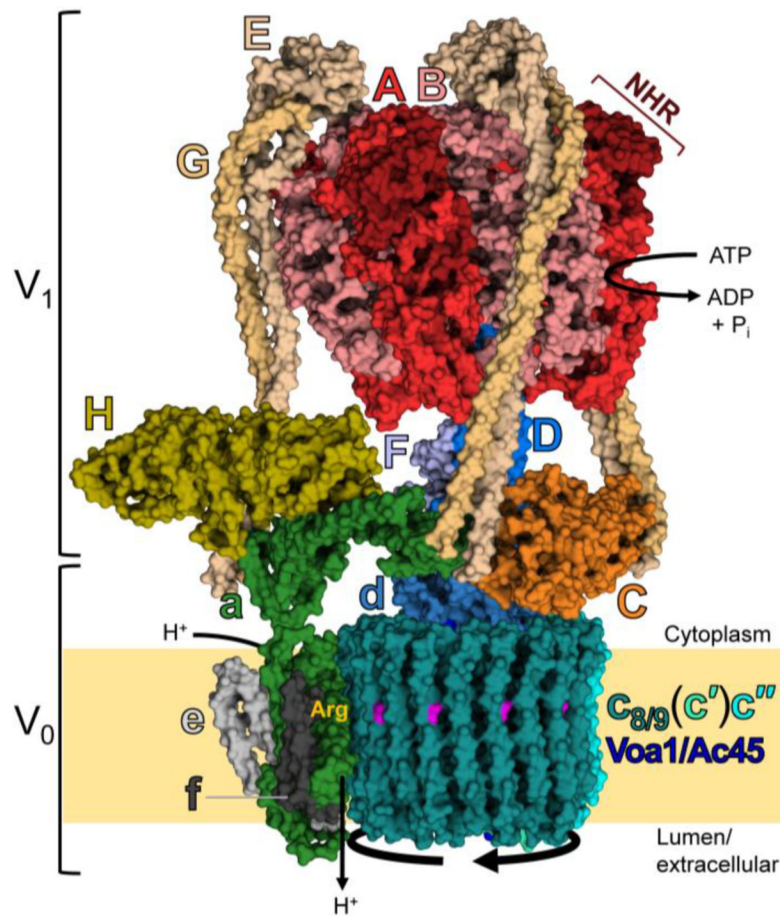


Fig. 1. Structure and mechanism of the V-ATPase.

The V-ATPase is organized into two large domains: the peripheral V₁ domain, which hydrolyzes ATP, and the integral V₀ domain, which transports protons. V₁ is composed of subunits A, B, C, D, E, F, G and H. The A₃B₃ hexamer contains nucleotide binding sites at each of the six AB interfaces, with three of the sites performing ATP hydrolysis. Subunit A also contains a 100 amino acid non-homologous region (NHR) which is not present in the ATP synthase β subunit. The V₀ domain is composed of subunits a, c, c' (in yeast), c'', d, e, f, and Voal (homologous to mammalian Ac45). The complex operates by a rotary mechanism, with ATP hydrolysis causing rotation of the central stalk and proteolipid ring. Protons enter an aqueous hemichannel in subunit a and protonate glutamate residues (magenta color) in each proteolipid subunit. After performing a full rotation, protons exit through a second hemichannel upon stabilization of the glutamate side chain by a critical arginine (yellow Arg) in subunit a. The complex is always oriented with V₁ in the cytoplasm, so proton transport is driven into luminal compartments or the extracellular space. This 6.6Å cryo-EM structure of the Stv1p-containing, yeast V-ATPase holoenzyme was determined by Vasanthakumar et al. (PDB ID: 6O7V) [46].

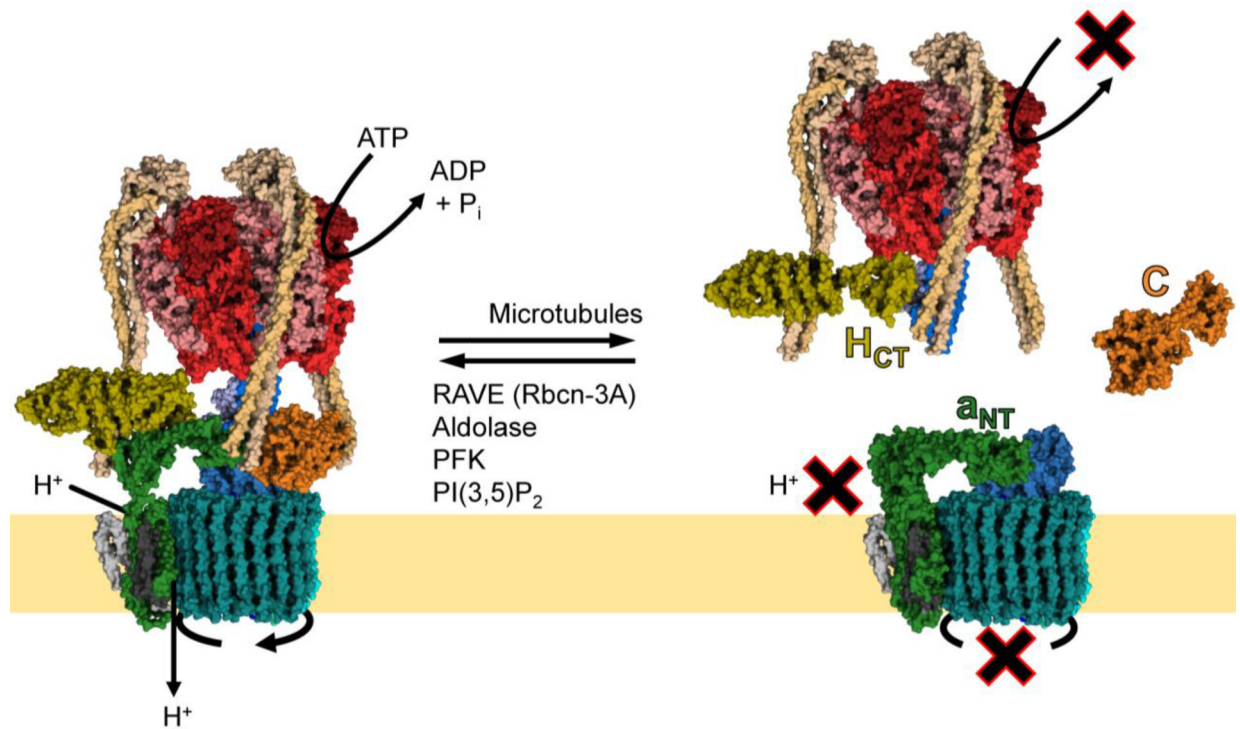


Fig. 2.
Regulated assembly of the V-ATPase.

An important form of V-ATPase regulation is the reversible dissociation of the V₁ and V₀ domains. In the disassembled state, free V₁ cannot hydrolyze ATP, and free V₀ cannot conduct protons. In free V₁, H_{CT} undergoes a large conformational shift, bringing it into contact with subunit F of the central stalk (PDB ID: 5D80) [45]. In free V₀, the N-terminus of Vph1p or Stv1p (a_{NT}) contacts subunit d of the central stalk (PDB ID: 6O7U) [46]. The only subunit that is released from either domain upon disassembly is subunit C. The process of reversible dissociation is rapid, does not require new protein synthesis, and occurs in response to diverse stimuli (see Table 1). In yeast, disassembly requires an intact microtubule network, while reassembly requires the RAVE complex, of which Rav1p is homologous to Rbcn-3A in higher eukaryotes. Other assembly factors common to yeast and higher eukaryotes include the glycolytic enzymes aldolase and phosphofructokinase (PFK). In yeast, PI(3,5)P₂ binds to Vph1p and promotes assembly of Vph1p-containing V-ATPase complexes.

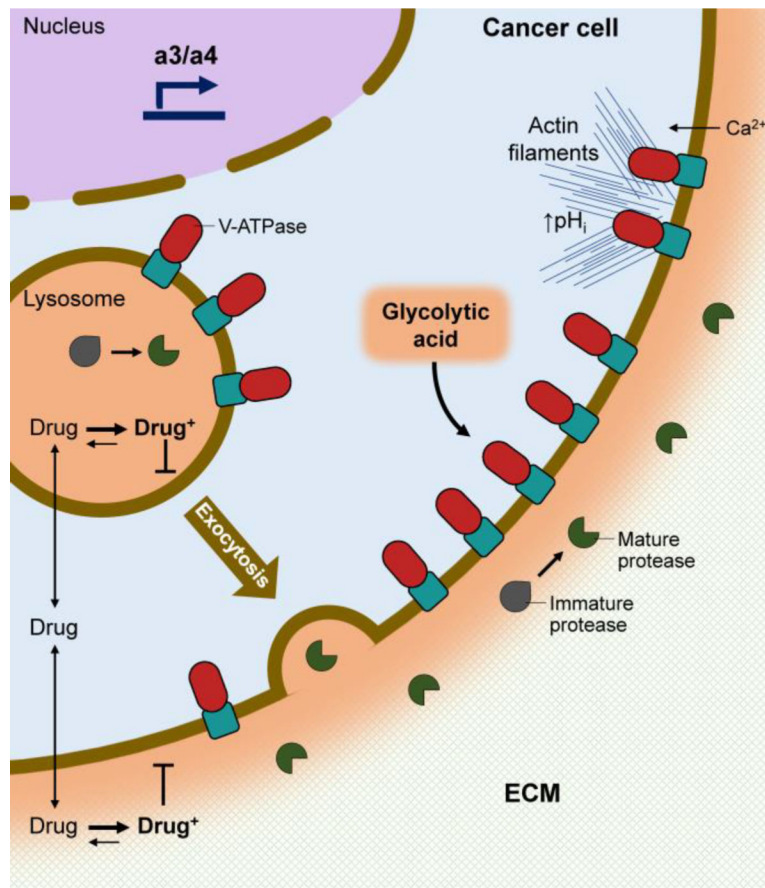


Fig. 3.

Function of V-ATPases in cancer cell survival, migration and invasion.

Cancer cells upregulate a3 and a4, which target V-ATPases to the plasma membrane, where they transport protons extracellularly. An acidic extracellular pH causes protonation of cancer drugs (Drug^+), which prevents their diffusion into the cell. Once in the cytosol, cancer drugs diffuse into acidic compartments, where they are retained upon protonation and prevented from reaching their intended targets. Plasma membrane V-ATPases also promote cancer cell survival by removing cytosolic acid produced from glycolysis. Plasma membrane V-ATPases may contribute to cell migration by creating regions of alkaline pH ($\uparrow\text{pH}_i$), which promote actin polymerization and branching near the plasma membrane. Alternatively, acidic extracellular pH may contribute to force generation at the leading edge through calcium influx. Finally, plasma membrane V-ATPases are thought to contribute to invasion by activating secreted proteases, which function to cleave extracellular matrix (ECM) components and to activate other secreted proteases such as matrix metalloproteases.

(Aldo) is occupied by fructose 1,6-bisphosphate (FBP), and promotes Ragulator GEF activity towards the Rags. mTORC1 may then be activated by Rheb if growth factors are present (shown in A). Under these conditions, AMPK present at the lysosome is inactive, because its upstream kinase LKB1 is retained in the cytosol in complex with the scaffolding protein Axin. When glucose is low, Aldolase is no longer occupied by FBP, and Axin-LKB1 is recruited to the lysosome where it activates AMPK. Axin-LKB1 binding also appears to inhibit the Ragulator-Rag complex and displace mTORC1. The V-ATPase is required for glucose dependent recruitment of Axin-LKB1 to the lysosome. V-ATPase assembly is also glucose-dependent, with glucose depletion leading to increased assembly, possibly as a way to increase autophagic flux.

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Table 1.

Known regulators of V-ATPase assembly.

Organism	Stimulus	Assem. change	Signaling pathways	Proposed function	Refs.
Yeast					
<i>S. cerevisiae</i>	Glucose starvation	↓	Ras/cAMP/PKA	Energy conservation	[83,87]
	Alkaline cytoplasmic pH	↑	?	PKA activation	[88]
	Alkaline extracellular pH	↑	?	pH homeostasis	[89]
	Vacuole neutralization	↑	?	Vacuole acidification	[90]
	Osmotic stress	↑	Fab1p (PIKfyve)	Na ⁺ homeostasis	[109]
Insects					
<i>M. sexta</i> midgut goblet cells	Molting	↓	PKA	Energy conservation	[84,92,93]
<i>C. vicina</i> salivary glands	Serotonin	↑	PKA	K ⁺ and Cl ⁻ efflux	[91–93]
<i>A. aegypti</i> malpighian tubules	Blood meal	↑	PKA	Solute and water efflux	[122]
Mammals					
MDCK, A549 and HeLa cells	High glucose	↑	PI3K	pH homeostasis	[114]
MDCK and A549 cells	Influenza infection	↑	PI3K/Akt MAPK	Viral entry	[115]
MA104 cells	Rotavirus infection	↑	PI3K/Akt MAPK	Viral entry	[116]
Primary mouse dendritic cells	Maturation	↑	PI3K/Akt/mTORC1	Antigen processing	[117,118]
Primary rat hepatocytes	EGF	↑	? (Did not test PI3K pathway inhibitors)	mTORC1 activation	[119]
HEK293T and LLCPK cells	Glucose starvation	↑	PI3K/Akt AMPK	Autophagic flux	[120]
HEK293T cells	Amino acid starvation	↑	Not PI3K or mTORC1	Protein degradation	[121]
HEK293T cells	Lysosome neutralization	↑	?	Lysosomal acidification	[121]
HL-1 cells	High palmitate	↓	?	Fatty acid uptake	[123]
Primary mouse hippocampal neurons	Synaptic vesicle recycling	↓	?	Vesicle exocytosis	[124]
		↑	?	Vesicle endocytosis	[124]